HIGH THROUGHPUT SCREENING USING FLUOROPHORE LABELED LIPID MEMBRANES AND FLUORESCENCE CORRELATION SPECTROSCOPY

Inventors: James H. Warner
2955 Arizona Ave.
Los Alamos, New Mexico 87544

Scott M. Reed
6207 NE 12th Avenue
Portland, Oregon 97211

Basil I. Swanson
3463 Urban
Los Alamos, New Mexico 87544

CITIZENS OF THE UNITED STATES

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STATEMENT REGARDING FEDERAL RIGHTS

This invention was made with government support under Contract No. W-7405-ENG-36 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to fluorescence-based assays to detect and monitor interactions between biochemical molecules. More particularly, the invention relates to an apparatus for and method of detecting binding between biochemical molecules by fluorescence correlation spectroscopy using fluorophore-labeled lipid membranes. Such a method and apparatus provide a means of rapidly screening large, combinatorial libraries to discover and quantify binding interactions.

BACKGROUND OF THE INVENTION

Various screening techniques have been developed to detect and monitor interactions between biochemical molecules and to identify biochemical molecules with unique features such as binding, inhibiting or catalytic functions. High-throughput screening is a process in which batches of compounds (e.g., molecular library elements) are tested for binding activity or biological activity against target molecules. The potential market for application of biosensor or high-throughput screening technologies is enormous and includes detection and diagnostics in the health care industry and environmental monitoring.

Screening techniques are generally based on detecting and monitoring a binding event between a recognition and target molecule. These molecules can be peptides, antibodies, antibody fragments, receptors, oligonucleotides, and oligosaccharides. These binding events include binding of a target molecule at a single binding site on a recognition molecule as well as binding at multiple sites (i.e., multivalent binding) of a target molecule by multiple recognition molecules.

Screening techniques incorporate a variety of detection methods. One highly sensitive detection technique is fluorescence correlation spectroscopy (FCS). FCS measures fluctuations in fluorescence intensity from a small number of fluorescently tagged molecules diffusing through a small detection volume (typically less than about 1 femtoliter) over a defined time range (typically microseconds to seconds). Diffusion of the tagged molecule through the detection volume produces a fluctuation in fluorescence intensity that is detected and discriminated from background noise by auto- or cross-correlation. The correlation function includes quantitative information about concentration and diffusion rates (e.g., molecular mass) of molecules in the sample. For example, the average time required for passage of a single fluorescent molecule through the detection volume is determined by its diffusion coefficient, which is related to the size of the molecule. Small, rapidly diffusing molecules produce rapidly fluctuating intensity patterns, compared with larger molecules that produce more sustained patterns of fluorescence.

FCS has been used in high-throughput screening assays to detect binding between a recognition molecule (e.g., a library element, such as an antibody) and a target molecule (e.g., an antigen). For example, a target molecule, such as an antigen with different binding epitopes can be used to screen a fluorescently labeled antibody library (e.g., a red fluorophore) for antibodies that recognize and bind the different antigen epitopes. Diffusion of the tagged molecule through detection volume produces a fluctuation in fluorescence intensity that can be detected and discriminated from background noise by auto correlation. Binding of a labeled antibody to the antigen can be detected by a shift from a rapidly fluctuating red fluorescence pattern to a more sustained or prolonged pattern of red fluorescence. However, the target molecule and recognition molecule must be sufficiently different in molecular mass, generally by about a factor of 10, in order for a shift in the fluorescence pattern upon binding to be detected. Thus, a need exists for a FCS screening assay that simultaneously labels a library element with

a fluorescent tag and significantly increases its effective size. Such a method is now provided by the present invention.

Another limitation in current high-throughput screening assays is that the binding between a target molecule and a recognition molecule often occurs at a single recognition site. Single-site binding events are often associated with issues such as low binding affinities and high on-off rates, and consequently the binding event is less stable and harder to detect. Multivalent binding events are generally more stable because they include multiple sites of interaction between a target molecule and recognition molecules. Multivalent binding events overcome the instability issues associated with single-site binding events, and are therefore easier to detect. This, a need exists for a FCS screening assay that is based on binding at multiple sites (i.e., multivalent binding) of a target molecule by multiple recognition molecules.

A final limitation of current screening methodologies lies in the fact that several protein receptors and oligosaccharides are water-insoluble and in nature are found segregated into the cellular membrane. The present invention enables the study of these water-insoluble molecules by surrounding such species with an appropriate lipophilic, biomimetric surrounding, i.e., a vesicle.

SUMMARY OF THE INVENTION

To achieve the foregoing and other objects, and in accordance with the purposes of the present invention, as embodied and broadly described herein, the present invention provides an apparatus including a light source, an objective, a first detector means for detecting light of a first defined wavelength range, a second detector means for detecting light of a second defined wavelength range, a first filter means for filtering light of a third defined wavelength range, a second filter means for filtering light of a fourth defined wavelength range, a support having a pinhole therein through which collected light from said objective is preferentially passed to said first detector means and said second detector means as opposed to out of focus scattered light, and, a transparent substrate for support

of a sample under investigation, said sample comprising membrane vesicles including a trifunctional linker molecule including a fluorophore.

The present invention further provides a method of detecting a binding event between biomolecules including admixing a target molecule including a first fluorophore and membrane vesicles including a trifunctional linker molecule, said trifunctional linker molecule including a second fluorophore, to form a sample, introducing a library of elements into said sample, each of said library elements having a binding affinity for said trifunctional linker molecule, and, screening said sample for fluorescence from said first fluorophore and said second fluorophore, such fluorescence indicative of a binding event between an element from said library of elements and said target molecule.

One embodiment of the present method involves use of fluorescence cross correlation to screen samples for a binding event by checking for correlations in the fluorescence light intensity measured by spectrally resolved detectors.

Another embodiment of the present method involves use of fluorescence correlation spectroscopy to screen samples for a binding event by examination of temporal durations that result from diffusion coefficients by target molecules bound to membrane vesicles.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates an example of a trifunctional linker molecule including a membrane anchoring group, a reporter group and a reactive or recognition group.

FIGURE 2(a) and 2(b) illustrate a schematic representation of a vesicle-based detection system.

FIGURE 3 illustrates a schematic representation of a detection apparatus for measurement of a sample using FCS.

FIGURE 4 illustrates a method of using the vesicle-based detection system in conjunction with the detection apparatus.

FIGURE 5(a) illustrates autocorrelation functions of unbound target and bound target and FIGURE 5(b) illustrates cross-correlation functions in the

presence and absence of a binding event between two molecules labeled with spectrally distinct fluorophores.

DETAILED DESCRIPTION

The present invention concerns a method of monitoring and screening molecular interactions. More particularly, the invention concerns a method of monitoring (e.g., chemical coupling reactions) and screening (e.g., display and combinatorial libraries) a sample by FCS using fluorophore-labeled lipid membranes.

The present invention provides a method to detect and to quantify binding events with target molecules. Such a method can be useful in the development of new pharmaceutical, therapeutic and sensing molecules. The method allows fast analysis times and miniscule sample requirements and can serve as a valuable tool in the screening of large combinatorial libraries for biological and chemical applications.

A preferred embodiment of the present invention involves use of a trifunctional linker as described in U.S. Patent No. 6,627,396, such a trifunctional linker including alkyl chain groups for anchoring or attachment to a substrate such as a lipid membrane substrate, a fluorescent moiety capable of generating a fluorescent signal, and a recognition moiety with a spacer group of a defined length, the recognition moiety for binding with a target molecule.

Fig. 1 illustrates an example of a trifunctional linker molecule **100** including a membrane anchoring group **110**, a reporter group **120** and a reactive or recognition group **130**.

Reactive group **130** (or alternatively, recognition group **130**) provides a chemically reactive site for coupling a recognition molecule, such as a peptide, to trifunctional linker molecule **100**. Other recognition molecules are well known by those skilled in the art and can be used as well.

Reporter group **120** can typically be any chemical or biochemical entity or label that yields an externally measurable output signal that can be correlated or assigned with a specific binding event. Suitable examples of such groups can

include fluorophores, isotopic labels or magnetic materials. Suitable fluorophores can be from the group of rhodamines, an intrinsically fluorescent protein, such as green fluorescent proteins (GFP), fluoresceins and the like.

Membrane anchoring group **110** provides mobile attachment of the entire trifunctional linker molecule **100** to a fluid surface of a membrane. Such a membrane anchoring group **110** can generally be any group that contains alkyl, alkenyl, alkynyl, and polyaromatic chains of carbon atoms containing from about 4 to about 30 carbon atoms. One preferred anchoring groups are long chain alkyl groups such as straight chain alkyl groups with 18 carbon atoms.

Trifunctional linker molecule **100** can be inserted into a lipid membrane, typically by adding a solution containing the linker molecule directly to a membrane lipid solution used to form vesicles. Trifunctional linker molecule **100** is incorporated into vesicles or micelles with reactive group **130** exposed on both the external and internal vesicle surfaces. Standard conjugation chemistry can be used to covalently attach a recognition molecule to reactive group **130**.

Fig. 2(a) illustrates a schematic representation of a vesicle-based detection system **200**. In one embodiment of the present invention, detection system **200** includes a membrane vesicle **210** and a target **220**. Target **220** is typically significantly smaller (i.e., an order of magnitude) than membrane vesicle **210**. Protein targets typically weigh from about 10,000 daltons to about 100,000 daltons and are approximately 10 nanometers (nm) in diameter. Suitable vesicles can then typically be 100 nm in diameter or larger. Larger sizes of vesicles can enhance the contrast in correlation time.

In this embodiment, target **220** further includes a fluorophore **230**. Target **220** is any target molecule of interest, such as a peptide or the like. In the presently illustrated embodiment, target **220** is a multivalent molecule. In an alternative embodiment, target **220** can have a single binding site.

Fluorophore **230** can be any fluorescent molecule that is distinct from reporter molecule **120** attached to trifunctional linker **100**. For example,

fluorophore **230** could be a green fluorescent molecule where reporter molecule **120** is a red fluorescent molecule.

The binding of target **220** by library element **240** can be detected and analyzed using FCS as described in reference to Fig. 3 and Fig 4. Fig. 3 illustrates a schematic representation of a detection apparatus **300** for FCS measurement of a sample. Detection apparatus **300** includes a light source **305**, an objective **310**, a first detector means **315**, a second detector means **320**, a first filter means **325**, a second filter means **330**, a support **332** having a pinhole **335** therein, and a substrate **340**. In operation, detection apparatus **300** further includes an aqueous sample droplet **345**, an excitation light beam **350**, a probe volume **355**, and an emission light beam **360**. Detection apparatus **300** can typically be an epi-fluorescence detection system, in which excitation light beam **350** travels through objective **310** to illuminate sample droplet **345** deposited upon substrate **340**. Substrate **340** can be any transparent substrate, such as a glass microscope slide or slipcover, which facilitates transmission of both excitation light beam **350** and emission light beam **360**. Emission light beam **360** from sample droplet **345** is subsequently collected and focused by objective **310**. Sample droplet **345** further includes a plurality of membrane vesicles **210**, targets **220**, and library elements **240**.

Light source **305** can be any conventional light source, such as a specific wavelength laser or a mercury vapor arc burner, which provides excitation light beam **350** suitable for the excitation of fluorophores within membrane vesicles **210** and target **220**.

Objective **310** can be any conventional converging lens, such as a 60x Nikon CFN plan apochromat, which focuses and transmits light. Probe volume **355** is the area of penetration of excitation light beam **350** from objective **310**, and represents the area of sample droplet **345** under FCS analysis.

Detector means **315** and detector means **320** can be conventional optical sensors, such as avalanche photodiodes (SPCM 200 PQ, from Perkin Elmer Optoelectronics, Quebec, Canada) for detecting light of a specific wavelength. In

the presently described embodiment, those wavelengths would be for green and red light, respectively.

Filter means **325** and **330** can be dichroic filters, e.g., conventional longpass optical filters, such as XF2010 (Omega Optical), that reflect light shorter than a certain wavelength, and pass light longer than that certain wavelength. For example, filter means **325** can be a filter that reflects wavelengths below 500 nm (where the wavelength of excitation beam **350** is at 496 nm). This filter then passes light above 500 nm, where fluorescence emission **360** occurs, i.e., the emitted fluorescence **310**. The emission light beam **360** can be further spectrally filtered by detector means **330**. This filter then reflects emission light beam **360** below 550 nm, and passes emission light beam **360** above 550 nm from sample droplet **345**.

Pinhole **335** formed within support **332** acts as a spatial filter to block scattered laser light and penetration of "out of focus" emission light beam **360** from sample droplet **345** through objective **310**. For example, "out of focus" emission light beam **360** is typically light that is not at the focal point of objective **310**. Pinhole **335** effectively provides penetration of "in focus" emission light beam **360** to detector means **315** and detector means **320** via filter means **330**.

Fig. 4 illustrates a method **400** of using detection system **200** in conjunction with detection apparatus **300** according to a preferred embodiment of the present invention. The preferred embodiment includes the use of two different fluorophores, i.e., a first fluorophore as a structural component of trifunctional linker **100** anchored in membrane vesicles **210** and a second fluorophore as part of targets **220**. Cross-correlation analysis of the two different fluorophores decreases background fluorescence from unbound membrane vesicles **210** and targets **220**, and increases the sensitivity of detecting a binding event. Method **400** generally includes the first step of providing membrane vesicles **410**, as previously described in reference to Figs. 2(a) and 2(b). The next step **420** is that of providing a target of interest. In step **420**, a target of interest **220** is provided. For example, target **220** can be added using standard microfluidic techniques to an aqueous

solution containing membrane vesicles **210** to form sample droplet **345**. Sample droplet **345** is typically about 4 microliters in volume. The next step **430** is that of introducing elements of a library. In step **430**, elements of a library (such as library element **240**) are provided. Library element **240** is introduced into sample interrogation region **345**, typically by standard microfluidic techniques, such as a stop/flow mechanism. Sample interrogation region **345** now includes membrane vesicles **210**, target **220**, and library element **240**.

The next step **440** is detecting a binding event. In step **440**, fluorescence detection is performed to detect a binding event. In a preferred embodiment, fluorescence detection is performed by FCS using detection apparatus **300** as described above. FCS is a standard technique commonly used in fluorescence-based detection assays.

In a typical FCS measurement, (i.e., autocorrelation or cross-correlation), fluorescence intensity is recorded over a time range from seconds to minutes. The time-dependent fluorescence intensity ($I(t)$) is then analyzed in terms of its temporal correlation function ($G(\tau)$), which compares the fluorescence intensity at time t with the intensity at $(t + \tau)$, where τ is a variable interval averaged over all data points in a time series. Mathematical auto- or cross-correlation of the data uses the following general formula:

$$G(\tau) = \langle \delta I_1(t) \delta I_2(t + \tau) \rangle / \langle I_1(t) \rangle \langle I_2(t) \rangle$$

The autocorrelation function measures the time-dependent fluorescence intensity ($I(t)$) for a single fluorophore where I_1 and I_2 are fluorescence intensity signals at different delay times. The autocorrelation function provides quantitative data on the concentration and size (i.e., diffusion rates) of molecules in a sample. The autocorrelation function further provides information on the interaction of two different molecules based on their differences in diffusion characteristics, as is shown and described further in Fig. 5(a).

The cross-correlation function measures the time-dependent fluorescence intensities of two spectrally distinct fluorophores where I_1 and I_2 are fluorescence intensity signals for different wavelengths, e.g., a green fluorescent signal and a

red fluorescent signal. The cross-correlation function provides quantitative information on the specific interactions between two molecules labeled with the spectrally distinct fluorophores. A cross-correlation signal is generated only when the two distinct fluorophores are detected in a single binding complex, as is shown and described further in Fig. 5(b). Cross-correlation analysis eliminates background fluorescence from non-interacting molecules and increases the sensitivity of detecting a binding event.

In a preferred embodiment, two different fluorophores are used to detect a binding event. The time-dependent fluorescence intensity of one fluorophore, such as a green fluorescent signal, and the time-dependent fluorescence intensity of a second fluorophore, such as a red fluorescent signal, are cross-correlated to determine whether the two fluorescent signals occur in the same binding event, i.e., whether they are co-localized to a single molecular complex.

In operation, sample droplet **345** is excited by excitation light beam **350** from light source **305**. Excitation light beam **350** is of sufficient wavelength to excite reporter molecule **120** (e.g., a red fluorophore) anchored in membrane vesicle **210** and fluorophore **230** (e.g., a green fluorophore) attached to target **220**. The movement via random diffusion of membrane vesicle **210** and target **220** into and out of probe volume **355** is detected by detector means **320** and detector means **315**, respectively. The time-dependent fluorescence intensity ($I(t)$) of each fluorophore is then analyzed in terms of its temporal correlation function ($G(\tau)$), as described above.

The next step **450** is the determination whether a positive binding event has occurred. If the determination of whether a positive binding event has occurred is yes, then the process proceeds to step **470**. If the determination is no, then the process proceeds to step **460**.

In step **460**, sample droplet **345** is removed from substrate, typically by standard microfluidic techniques, such as a stop/flow mechanism. Sample droplet **345** can then be discarded and the process returned to the beginning with step **410**.

In step **470**, sample droplet **345** is removed from substrate, typically by standard microfluidic techniques, such as a stop/flow mechanism. Sample droplet **345** is then stored for isolation and analysis of the particular library element **240**. In one embodiment, the output is collected in the capillary of a tube. In an array embodiment, the droplet could be suctioned off with a pipette or capillary for further analysis. A next step **480** could then be used to examine for additional binding events.

Fig. 5(a) illustrates autocorrelation functions of unbound target **220** and bound target **220**. The autocorrelation function measures the time-dependent fluorescence intensity for a single fluorophore (e.g., only target **220** is fluorescently labeled) and provides information on the interaction of two different molecules based on differences in their diffusion characteristics. Detection of a binding event between target **220** and a recognition molecule, such as an antibody, requires that the interacting components differ in molecular mass by at least a factor of about 10. The initial amplitude of the autocorrelation function is inversely proportional to the number of targets **220** in probe volume **355**. The autocorrelation function decays from its initial value with a time-dependence that is determined by the molecular diffusion rates of target **220**. Target **220**, typically a lower molecular weight molecule, exhibits a faster autocorrelation decay in its unbound state (approximately 0.1 milliseconds) and a slower autocorrelation decay (about 5 milliseconds) when bound to a membrane vesicle.

Fig. 5(b) illustrates cross-correlation functions in the presence and absence of a binding event between two molecules labeled with spectrally distinct fluorophores. For example, target **220** is labeled with a green fluorophore such as a green fluorescent protein (GFP) and membrane vesicle **210** is labeled with a red fluorophore such as Texas Red. In the absence of a binding event between target **220** and membrane vesicle **210**, no cross-correlation signal is generated. A cross-correlation signal is generated only when the two distinct fluorophores are detected in a single binding complex, i.e., target **220** is bound to membrane

vesicle **210**. The cross-correlation signal is independent of diffusion rates (i.e., molecular mass) of the interacting molecules.

Although the present invention has been described with reference to specific details, it is not intended that such details should be regarded as limitations upon the scope of the invention, except as and to the extent that they are included in the accompanying claims.